



Experiment title: **Studies of cadmium and mercury ligand environment in fungal endophytes to improve the outcomes of phytostabilisation of extremely metal contaminated sites**

Experiment number:
LS- 2275

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Report:

Cadmium (Cd) and mercury (Hg) are among the most toxic and hazardous metals found in environment. They accumulate in food webs with biomagnification pattern at successive trophic levels, posing threat to human and animal health [1]. Phytostabilization is an in situ technology involving soil amendments and metal-tolerant plants to establish a ground cover that can reduce transfer of metals to air, surface and ground water and reduce soil toxicity. Efficient mutual symbiosis between suitable fungal endophytes and plants may be crucial for the survival of plants in metal polluted areas through improving their nutrient and water acquisition and providing protection from metal toxicity [2]. Among the fungal endophytes, mycorrhizal fungi and dark septate endophytes (DSE) form the most intimate relationships with plants. Especially DSE fungi have been found in great abundance in the roots of the plants from metal-enriched sites [3,4], which is probably a result of greater metal tolerance due to the presence of melanin in their cell walls [5]. Fungal melanin has many free carboxylic and hydroxylic functional groups that have high affinity to bind heavy metals, such as Cu, Cd and Hg [3], therefore preventing their uptake into fungal cells, as well as further transport into the plant tissues. Recently we have isolated DSE fungal strains from the roots of willows growing at Cd, Pb and Zn polluted site in Žerjav, Slovenia [6] and in Hg polluted site in Idrija. The strains showed various level of tolerance to metals in vitro, therefore the aim of this study is a) to estimate the contribution of melanin synthesis to Cd and Hg tolerance in the presence (or absence) of melanin synthesis inhibitor, b) to investigate Cd and Hg ligand environment (either -O or -S) in fungal mycelium and c) to test the relation between metal accumulation in mycelia and the level of melanin synthesis using high performance liquid chromatography.

Within the beamtime we analysed Cd, Hg, Pb, Cu, Zn, Co, Fe and Mn ligand environment in selected plant species and reference metal complexes. For determination of metal ligand environment we used freeze-dried plant material in order to ensure high enough metal concentrations to obtain good signal to noise ratio [6]. After harvesting the plants were rapidly frozen in liquid nitrogen and freeze-dried. The dried material was homogenised in a mortar with liquid nitrogen and pressed into self standing pellets which were mounted on the He cryostat holder of the beamline. XANES and EXAFS spectra were measured in cryo conditions.

The EXAFS and XANES spectra were measured in fluorescence or transmission detection mode, depending on the concentration of the investigated element in the sample, at BM23 beamline. For Cd K-edge (26,711 eV) XAS a double crystal Si(311) monochromator with 2 eV resolution was used, while in the energy region of region of Hg and Pb L₃-edge, and Cu, Zn,Co, Fe and Mn K-edge a Si(111) monochromator with energy resolution of about 1 eV at 10 keV was used. Higher harmonics were eliminated by a flat rhodium coated mirror placed after the monochromator. The intensity of the monochromatized beam was monitored with three ionization detectors, filled with krypton to a pressure of 120 mbar, 420 mbar and 450 mbar, respectively, for the Cd K-edge measurements. In the case of Hg and Pb L₃-edge measurements, the cells were filled with argon to a pressure of 220 mbar, 754 mbar, and 1600 mbar, respectively. For Cu and Zn K-edge measurements, the cells were filled with argon to a pressure of 90 mbar, 300 mbar, and 1160 mbar, respectively. For Mn, Fe, and Co K-edge measurements, the cells were filled with 830 mbar of N₂, 135 mbar of Ar, and 505 mbar of Ar, respectively. In all cases He was added to a total pressure of 2 bars. Samples were placed between the first pair of detectors, and the reference metal foils between the posterior pair, to check the stability of the energy scale and to obtain the exact energy calibration. The absolute energy reproducibility of the measured spectra was ± 0.02 eV. A thirteen segment germanium solid state detector was used to collect fluorescence signal from the plant samples.

The absorption spectra were measured within the interval $[-250$ eV to $1,000$ eV] relative to the investigated absorption edge. In the XANES region, equidistant energy steps of 0.3 eV were used, while for the EXAFS region, equidistant k-steps ($\Delta k \approx 0.03 \text{ \AA}^{-1}$) were adopted, with an integration time of 1 s/step. The absorption spectra of two or three identical runs were superimposed to improve the signal-to-noise ratio and to check the stability and reproducibility of the detection system. The XANES and EXAFS spectra were analysed with the IFEFFIT package [7].

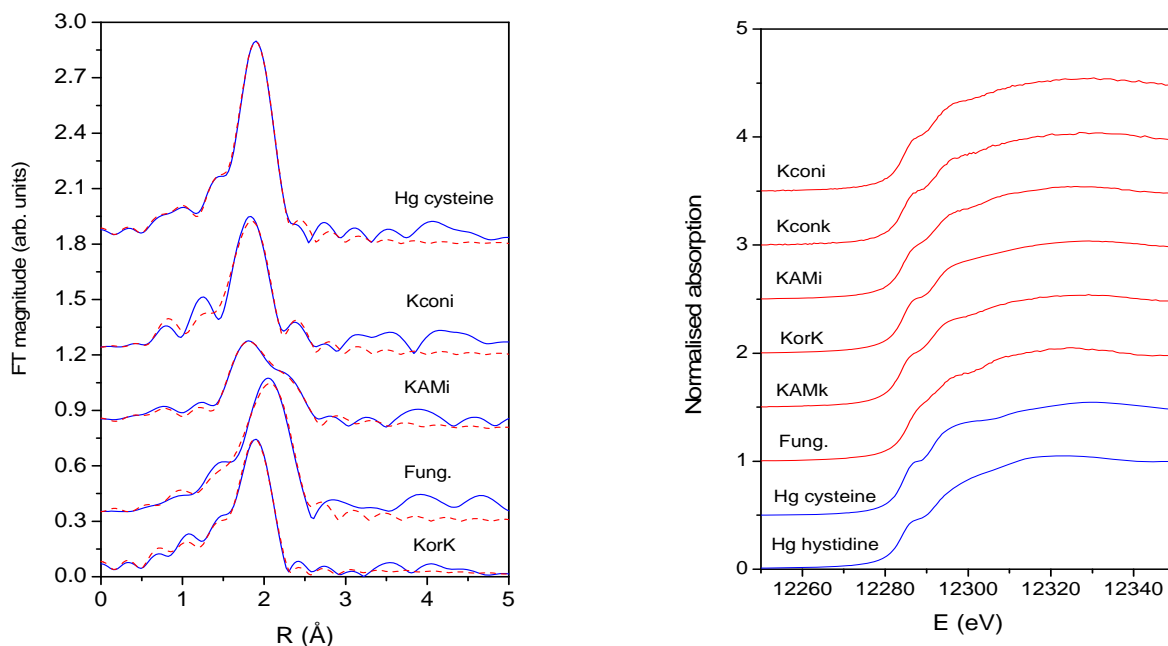


Figure 1: **Left:** FT magnitude of k^2 weighted Hg L₃-edge EXAFS spectra measured on plants treated with Hg and on reference complex Hg-cysteine (solid line – experiment, dashed line – best fit EXAFS model). **Right:** Hg L₃-edge XANES spectra measured on plant tissues and on reference Hg compounds.

The Hg L₃-edge XAFS spectra were measured on fungal mycelia and on the roots of the maize plants. In addition, organic Hg complexes with histidine, cysteine and cellulose were prepared as standards for N-, S-, and O-ligand environments, respectively. Fungal isolates originating from the vicinity of Idrija mercury mine have been used to inoculate maize plants exposed to 50 mg kg^{-1} of Hg [sample KAMi]. The protective effect of the inoculation is judged against non-inoculated plants [KorK], the plants inoculated with commercial fungal strains [KAMk], and the filtered commercial and Idrija inoculums retaining only bacterial isolates [Kconi and Kconk]. In addition, dark septate fungal endophytes were exposed to Hg in the same way [sample Fung]. Due to low concentration of the absorbed metal, the Hg L₃ XAFS signal could be, even with prolonged collection time, used only to $k \sim 10 \text{ \AA}^{-1}$, allowing the determination of the immediate ligand atom

and providing some hints about the neighbours in the 2nd shell. To improve the identification, samples of Hg II compounds or complexes with biochemically important ligands O, N, S, and P were analyzed for typical inter-atom distances.

Using *ad hoc* two-shell Feff [8] models with neighbours at appropriate distances we show that in most plant samples Hg is prevalently bound to sulphur at 2.35 Å as in cysteine or some other thio-compound, with possible minority ligands (N or O) at ~ 2.05 Å (Fig. 1). The samples Fung and KAMk show a more complex Hg neighbourhood with a relatively strong component between 2.50 and 2.70 Å, compatible with a remote S neighbour or a PO₄ group. Binding to carboxylic acids is, judging by comparison with XAFS spectra of Hg-acetate and Hg-cellulose complex, limited to amounts at the detection limit (10 %).

Metal cation bonding in the plant tissue can be identified also from the characteristic shape of the edge features and pre-edge resonances. Different local environments of the cation result in different edge profiles and pre-edge lines in the XANES spectra. In plant cells metal cations are expected to be bound to different complexes. Most probable metal complexes are prepared and measured to obtain the set of reference XANES spectra for the XANES analysis. The relative amount of metal complexes in plants is analysed with the linear combination fit method [7], where the XANES spectra measured on plants are modelled with a linear combination of the XANES spectra of the reference compounds.

In cases of Zn K-edge XANES measured in plants treated with Zn, major part of Zn is found to be coordinated to citrate (Zn-O-C coordination), while other Zn ligands are histidine (Zn-N-C coordination), cistein (Zn-S-C coordination) and in some cases nicotinamine. An example of the fit is shown on Fig. 2. All other combinations with the rest of the reference spectra were rejected in the fit procedure or lead to fits with significantly higher R-factor, which measure the quality of fit.

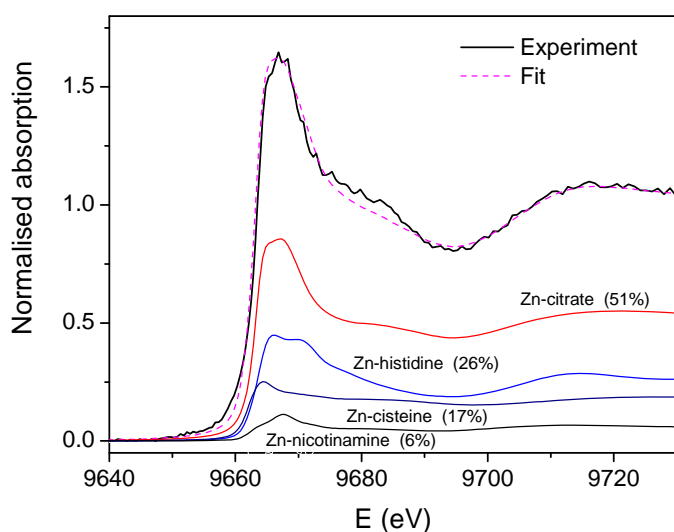


Figure 2: The Zn K-edge XANES spectrum measured on roots of Zn treated plants. Black solid line - experiment; magenta dashed line – best-fit linear combination of XANES profiles of Zn-citrate (51%), Zn-histidine (26%) and Zn-cysteine (17%) nad Zn-nicotinamin (6%). Fit components are shown below.

The quantitative XANES and EXAFS analysis is in progress to obtain ligand environment of the metal cations in plant tissues. The results are prepared for publication.

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